Pre-Made MicroRNA-Enriched cDNAs

Cat. # RA500A-1 – RA509A-1

User Manual

Store kit at -20°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 1-061019)

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I. Introduction and Background

A. Overview

This manual provides details and information necessary to use the Pre-made MicroRNA-Enriched cDNAs for cloning of selected small gene transcripts processed by RNase III. To ensure optimal results, please read the entire manual before using the reagents supplied with this kit. The kit contains cDNA sufficient for several hundred quantitative PCR experiments or 5 cloning reactions.

B. Importance of MicroRNAs and Other Small Non-Coding RNAs

The field of non-coding RNAs has gained increasing attention in recent years, particularly due to the discovery of micro RNAs (miRNA). Micro RNAs are short (typically 19-24 nucleotides) single stranded RNAs that regulate the expression of target genes by interacting with complementary sites in the 3' UTR of the target mRNAs and inhibiting translation. miRNAs are a conserved group of non-coding RNAs with very important regulatory roles.

Mature miRNAs are excised from stem-loop precursors, which are themselves transcribed as part of longer primary transcripts. These primary miRNAs appear to be first processed by the RNAse Drosha in the nucleus, after which the precursor miRNAs are exported to the cytoplasm where the RNAse Dicer further processes them. These enzymes are also involved in the generation of mature small inhibitory RNAs (siRNA) from exogenously transferred double stranded siRNA precursors.

The current method for identifying novel miRNA molecules involves many steps and can take several days to complete. The method of Lagos-Quintana, et al requires three size selection steps of small RNA, two ligation steps—each requiring gel electrophoresis and RNA extraction steps—a reverse transcription step, followed by PCR amplification. SBI's Pre-made MicroRNA-Enriched cDNAs drastically reduces both the labor and time required to investigate miRNA populations. The kit utilizes an innovative set of degenerate adaptors to specifically recover RNaseIII processed molecules, including mature miRNAs as well as larger stem loop precursor molecules that current methods miss.

Recently, previously unknown germline specific classes of miRNA-like molecules were identified in mouse testes and mouse oocytes, illustrating the need for continued and in depth miRNA discovery efforts across a wide range of tissues. These facts taken together demonstrate an ever-increasing need for simple, robust, and sensitive methods that enable discovery and quantitation of microRNAs and their precursors.

C. Protocol Overview

A complete protocol workflow and data analysis schematic is shown in Figure 1.

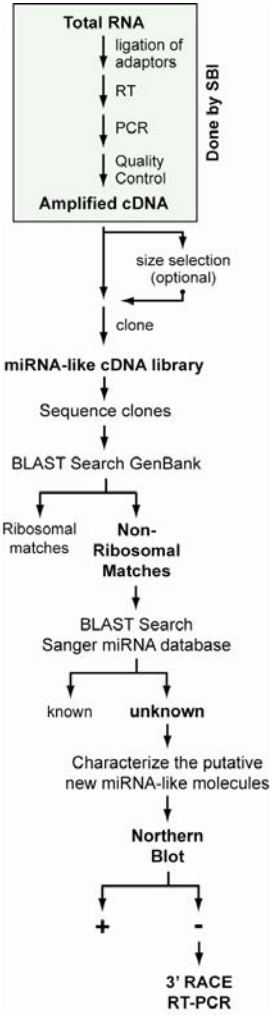

Fig. 1. Workflow schematic for a typical MicroRNA discovery experiment. Note that the initial cDNA amplification steps shown in the box have been completed by SBI.

SBI's small RNA amplification system includes 3 steps, which can be completed in less than 4 hours:

1. A degenerate adaptor mixture is ligated to both the 5'-end and 3'-ends of total RNA. Our use of chimeric DNA/RNA adaptors unique cocktail of enzymes, and optimized reaction conditions ensure efficient ligation. Figure 2 describes this process in detail, and Table 1 contains the sequences for the adaptor.

2. Reverse transcription of the RNA using a primer complementary to the attached adaptor.

3. PCR amplification of the cDNA.

These first three steps of ligation, reverse transcription, and PCR amplification have been performed at SBI. The amplified cDNA provided in the Pre-made MicroRNA-Enriched cDNAs is ready for direct TOPO-TA cloning (Invitrogen Cat. # K4500-01) with no additional purification necessary. If desired, the amplified cDNA can also be cloned by standard cloning protocols.

Upper strand adaptor

1. 5'-(P) ACTCTGCGTTGATACCACTGCTT-3'

Lower strand adaptors

2. 3'-rNrTrGrAGACGCAACTATGGTGACGAA(NH2)-5'

3. 3'-rNrNrTrGrAGACGCAACTATGGTGACGAA(NH2)-5'

4. 3'-rNrNrNrTrGrAGACGCAACTATGGTGACGAA(NH2)-5'

5. 3'-rNrNrNrNrTrGrAGACGCAACTATGGTGACGAA(NH2)-5'

RT and PCR primer

6. 5'-AAGCAGTGGTATCAACGCAGAGT-3'

Table 1. Sequences of adaptors and primers used in this study**.**

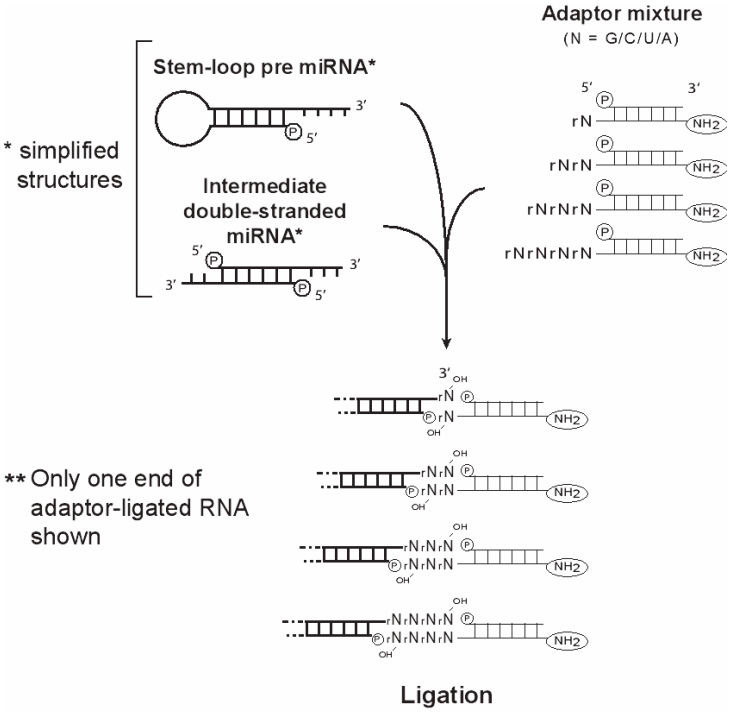

Fig. 2. Depiction of degenerate adaptor ligation step. A pool of double-stranded degenerate adaptor, which have a variable 3' overhang is ligated to total RNA.

The expected size distribution and banding pattern of amplified cDNA is shown in Figure 3 below. The size of the amplified cDNA can be refined through the use of a size selection step (see Part II, step C, and Figure 4 in this manual). While optional, we have found that this step can reduce the number of background clones resulting from ribosomal and transfer RNA transcripts, as well as increase the number of mature miRNAs recovered.

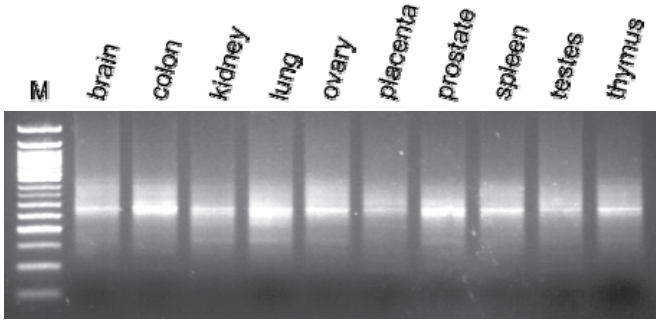

Figure 3. Size distribution of amplified cDNA from different Pre-made MicroRNA-Enriched cDNAs.

D. List of Components

Each Pre-made MicroRNA-Enriched cDNA Kit provides enough material to perform 5 cloning and screening experiments (5 reactions).

20	µl	Pre-made MicroRNA-Enriched cDNAs (25 ng/µl)
60	µl	RT and PCR Primer (20 µM)

The kits are shipped in blue ice and should be stored at -20°C upon receipt. Properly stored kits are stable for 1 year from the date received.

E. Additional Required Materials

- TOPO TA Cloning Kit (Invitrogen, Cat. # K4500-01)
- Microbiological plates with LB-agar supplemented with 50 µg/ml ampicillin
- Thermocycler (with heated lid)
- 3.0-3.5% Agarose Gel in Tris-Borate EDTA (TBE) or Tris-Acetate EDTA (TAE) Buffer
- DNA Size Ladder with markers from 50 to 2,000 bp (Bio-Rad AmpliSize™ DNA Ladder; Cat. # 170-8200)

F. Procedural Guidelines

- Before dispensing, completely thaw all reagents and vortex to mix thoroughly.
- Briefly centrifuge each mixture once to ensure there is no solution left on the sides/lid of the tube.
- When setting up multiple reactions, we recommend that you prepare a master mix.

II. Protocol

A. TOPO TA Cloning

When using Invitrogen's TOPO TA Cloning Kit, follow the manufacturer's instruction. We recommend using 4 ul of amplified cDNA in a 6 ul TOPO cloning reaction.

B. Sample Screening Guidelines for Identification of Putative miRNA or miRNA-like Molecules in MicroRNA-Enriched cDNAs

1. After cloning the cDNAs into TOPO cloning vector (Invitrogen) select white colonies and transfer them to 96-well flat bottom microtiter plate with 100 μl LB media supplemented with 100 μg per ml ampicillin. Grow colonies in the shaker at 37^{o} C for two hours.

2. To analyze size of the insert prepare PCR master mix for number of reaction equal to number of the clones need to be analyzed.

	1 Rxn		**10 Rxn**	
RNase-free Water	16.0	μl	160	μl
10X PCR Buffer	2.0	μl	20	μl
50X dNTP Mix (10 μM each)	0.4	μl	4	μl
RT and PCR Primer	0.5	μl	5	μl
PCR Polymerase	0.5	μl	6	μl
Total volume	19.4	μl	194	μl

3. Aliquot 19.4 μl of PCR master mix to 96-well PCR plate. Transfer 0.6 μl of cell culture directly from 96-well flat bottom plate to 96-well PCR plate. Seal the top of PCR plate and place into the PCR thermal cycler.

4. Place the reactions in a thermal cycler, and cycle using the following program:

   - 95°C for 2 min
   - 95°C for 20 sec
   - 68°C for 25 sec

   ] 25 cycles

   - 15°C hold

5. After amplification, analyze 2.5 μl of each reaction on a 3.0-3.5% agarose gel in 1X TBE or TAE Buffer. Include a DNA size ladder with markers in the range of 50-2,000 bp (*e.g.*, Bio-Rad AmpliSize™ DNA Ladder).

6. Select clones with desired size of the insert. **Note**: RT-PCR primer will add approximately 50 base pairs (bp) to the size of the insert, i.e.

clones with insert of 70-85 bp will be more likely contain mature form of miRNA or miRNA-like molecules. Larger inserts may contain precursors of miRNA or miRNA-like molecules.

7. Grow cells with desired size insert for plasmid purification followed by sequencing. For sequencing, we recommend purifying plasmid from 3ml of overnight culture using QIAprep plasmid mini purification kit (Cat. # 27104).

8. After sequencing, identify flanking vector sequences containing BamHI sites (GGATCC). Trim adaptor sequences from the both sides of the insert (24 nucleotides for the adaptor). Remaining internal sequence is the actual insert sequence, which requires further analysis.

9. Use this sequence for the BLAST search against GenBank (http://www.ncbi.nlm.nih.gov). Choose the database from the appropriate organism for your search to eliminate unrelated hits. For clones from human sources use human database, for mouse sources of RNA use mouse database and so on. The first GenBank BLAST search serves to rapidly eliminate background sequences derived from rRNA and tRNA and their degradation products. When analyzing results from the first BLAST be aware that some mRNA may have extensive homology (up to 180-200 nt) to rRNA and omitting clones containing these inserts may limit number of potential microRNA sequences for discovery. In parallel, you can BLAST search this sequence against mRNA database (RefSeq of GenBank) which can help confirm results from the initial GenBank BLAST search. The RefSeq BLAST search cannot substitute for the GenBank BLAST search because RefSeq does not contain known miRNA or other known small non-coding RNA entries.

10. When analyzing sequences, give the most attention to the hits with short sequence homology (usually 18-35 nt) to mRNA or 3'UTR of mRNA in plus/minus orientation to mRNA sequences. These are potentially new microRNA-like sequences.

11. An important criterion for miRNA validation is evidence of miRNA biogenesis. This typically requires the identification of pre-miRNA stem loop precursors. For secondary structure analysis use the whole insert sequence as derived in step 9, as well as the complementary strand. For this purpose we utilized the Sfold tool, available online at http://sfold.wadsworth.org (12). Folding analysis was performed on both the direct sequence read from the clone and the complementary sequence, from which select the best stem-loop structure with lowest minimum free energy.

12. Complete bioinformatics analysis of the data gathered in step 10 together with predicted secondary structure from step 11 can reduce number of potential new microRNA or microRNA-like sequences that require further experimental analysis. Based on the definition of

biogenesis of miRNA sequences, the mature miRNA should be in a stem of the stem-loop structure and lie on one side of the stem.

13. Northern blotting is still the most important criterion to categorize a new sequence as a miRNA. **Note**: We recommend that the oligonucleotide probes used for Northern blot hybridization are designed to be complementary to mRNA sequence or 3'-UTR sequence of mRNA (this is with assumption that this particular mRNA is the target for new miRNA). In case of lack of the signal in expected region you can try to use sequence homologous to mRNA sequence (this is in case if mRNA is the precursor in miRNA maturation and the target is the different mRNA sequence which is not picked up in BLAST search). **Note**: Lack of signal in the expected range may be due to low abundance level of particular putative miRNA in total RNA and the low sensitivity of Northern blot hybridization.

14. RT-PCR can be used to reduce the number of sequences subjected to Northern blotting. RT-PCR is much faster and more sensitive than Northern blot analysis. Since the miRNA and miRNA-like molecules are very small, a modified form of RT-PCR termed 3' RACE RT-PCR can be performed. Both Northern blotting and RT-PCR analyses are based on the specific hybridization of a small synthetic oligonucleotide to the target RNA, either as a probe or a primer. Northern blot analysis can estimate the size of the miRNA directly, while RT-PCR can also be used to estimate the size of the miRNA by gel electrophoresis. In this case the size is estimated by subtraction of the length of the adaptor from the size of the RT-PCR products. Furthermore, both methods can identify precursor forms of miRNA. The method of Shi and Chiang (11) can be adapted for RT-PCR analysis of putative miRNAs. In this method, a poly-A tail is added to the 3'-end of the RNA with poly-A polymerase. An oligo-dT containing adaptor is annealed and used for first strand cDNA synthesis. Following reverse transcription, a primer complimentary to the adaptor was used as a reverse RT-PCR primer. The forward primer consisted of the miRNA-like sequence itself. We recommend using 0.5 μg of the total RNA as starting material. For tailing of total RNA use poly-A polymerase (we recommend Epicentre poly-A-polymerase Cat. # PAP5104H) and follow manufactures protocol.

15. To analyze the presence of putative miRNA sequence in total RNA prepare PCR master mix for number of reaction equal to number of the different sequences to be analyzed. For PCR we recommend Klentaq LA DNA Polymerase from Sigma (Cat.# D5062).

	1 Rxn		10 Rxn	
RNase-free Water	15.3	µl	153	µl
10X PCR Buffer	2.0	µl	20	µl
50X dNTP Mix (10 µM each)	0.4	µl	4	µl
Forward RT-PCR Primer	0.4	µl	4	µl
Reverse RT-PCR Primer	0.4	µl	4	µl
cDNA from step 14	1.0	µl	1	µl
PCR Polymerase	0.5	µl	5	µl
Total volume	20.0	µl	200	µl

16. Place the reactions in a thermal cycler, and cycle using the following program:

    - 95°C for 2 min
    - 95°C for 20 sec
    - 60°C for 25 sec

    ] 25 cycles

    - 15°C hold

17. After amplification, analyze 2.5 µl of each reaction on a 3.0-3.5% agarose gel in 1X TBE or TAE Buffer. Include a DNA size ladder with markers in the range of 50-2,000 bp (*e.g.*, Bio-Rad AmpliSize™ DNA Ladder).

18. If you confirm by Northern blot or by 3' RACE RT-PCR analysis that the sequence is very likely to be new miRNA or miRNA-like molecule you may want to examine the distribution and maturation stages of this RNA sequence in different tissues or different cell lines with different treatments (inducing/repressing conditions). For this purpose it is very convenient to use 3' RACE RT-PCR. For example see Fig.7.In many cases miRNAs display different patterns in different tissues which can be additional indication of discovery of new non-coding small RNA molecules.

C. Size Selection of cDNA from Pre-made MicroRNA-Enriched cDNAs (optional)

1. Amplified cDNA from the Pre-made MicroRNA-Enriched cDNAs can be size selected for low molecular weight products. For purification, use 5 µl of cDNA solution. Apply sample on 3-3.5% agarose with 1X TAE. Include a DNA size ladder with markers in the range of 50-2,000 bp. Cut gel between 50 to 120 bp (even if you do not see any smear in this region). Purify cDNA from the agarose using Qiagen Gel Purification kit (Cat.# 28704), following the manufacturer's protocol. Use all of the extracted material for additional PCR amplification. Add the following:

53	µl	RNase-free Water
10	µl	10X PCR Buffer
2	µl	50X dNTP Mix (10 µM each)
2	µl	PCR Primer
3	µl	PCR Polymerase
100	µl	Total volume

2. Place the reactions in a thermal cycler, and cycle using the following program:

   - 95°C for 2 min
   - 95°C for 20 sec
   - 68°C for 25 sec

   ] 25 cycles

   - 15°C hold

3. Place the reactions in a thermal cycler, and cycle using the following program:

4. After amplification, run 2.5 µl of each reaction on a 3.0-3.5% agarose gel in 1X TBE or TAE Buffer. Include a DNA size ladder with markers in the range of 50-2,000 bp (*e.g.*, Bio-Rad AmpliSize™ DNA Ladder). You should see results similar to those shown in Figure 4. The typical yield is 2-3 µg cDNA. You can continue adding two-cycle increments until you see the sufficient product from your amplification reaction, however, you should not exceed 30 cycles.

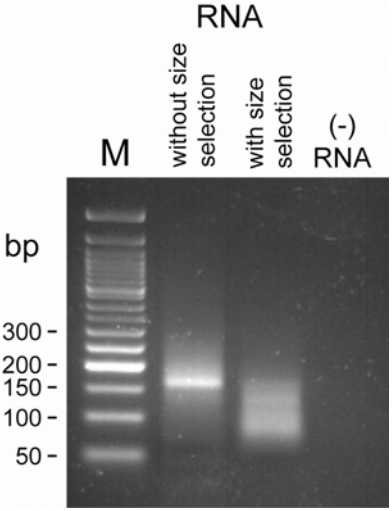

Figure 4. Distribution of cDNA after amplification before and after size selection.

III. Troubleshooting

A. No Colonies after TOPO TA cloning

- Try to use fresh PCR reaction product to make sure TOPO TA cloning kit is working.
- Try to amplify cDNA from the kit (25 ng or 1 μl) for 5 PCR cycles using PCR primer from the kit and repeat cloning.

B. No Product with PCR primer during colony screening

- If you do not amplify product, or see non-specific products (smearing) with PCR primers during colony screening, there may be a problem with your PCR reagents. Try to amplify with M13 forward and reverse primers. If the PCR still fails to generate a product after 35 cycles, try using new PCR reagents and enzyme.
- We typically use Klentaq LA DNA Polymerase from Sigma (Cat.# D5062).

IV. References

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V. Appendix

A. Data Generated using the Pre-made MicroRNA-Enriched cDNAs

The data below demonstrates that the cloning and amplification techniques used in SBI's Pre-made MicroRNA–Enriched cDNAs enrich for the majority of known miRNAs. Additionally, data is presented that demonstrates the power of the technique to identify novel RNaseIII processed miRNA-like transcripts.

A robust miRNA amplification procedure should both maintain the presence of individual miRNAs within the population, and enrich specifically for the target miRNA molecules. In our experiment, we have demonstrated that the majority of known miRNAs (13 of 14) were amplified and maintained throughout the amplification procedure.

It should be expected that our amplification process will enrich for miRNA-like sequences. We examined this question by comparing the number of PCR cycles needed to amplify a miRNA and the number needed to amplify a segment of the 5S rRNA transcript. Before amplification, the difference in the number of cycles needed to amplify the 5S rRNA and miR-1 was +9. After amplification the number of cycles was -1. The difference was about 10 cycles, which suggests an enrichment factor of about 1,000. Care was taken to avoid the plateau phase of the PCR amplification by limiting cycle numbers. Results are shown in Table 2.

	Cycles of PCR 5S rRNA/miR-1		Difference in cycles (miR-1 - 5S rRNA)
Before amplification	23	32	+9
After amplification	23	22	-1

Table 2. Estimation of miR-1 enrichment factor after cDNA amplification

Using techniques described in detail in SBI's MicroRNA Discovery Kit (cat# RA410A-1), we amplified and cloned the RNaseIII processed RNA fraction from total RNA without any size selection. The size of the cloned cDNA inserts ranged in size between about 100 and 300 bp. In the first small survey, 60 clones were selected for further examination by sequencing. BLAST searches against GenBank found that approximately 60% clones were derived from rRNAs or tRNAs and were omitted from further study. The remaining clones were found to

have homologies of between 18-29 nts to sequences in GenBank. While not 100% predictive, it is expected that a true miRNA will have a detectable level of similarity with its corresponding target transcript. miRNAs regulate mRNA translation and/or mRNA stability through hybridization with their target mRNA transcripts. Ultimately the set of non-rRNA/tRNA clones were found to contain four previously known microRNAs, and seven new miRNA-like sequences which are discussed in more detail below.

Four cDNA clones (4, 7, 10, and 13) were identified to contain miRNA-like sequences. Clones 10 and 13 each contained more than one miRNA-like sequence, for a total of seven individual miRNA-like sequences (4, 7, 10a, 10b, 13a, 13b, 13c). Multiple miRNAs in the same precursor, referred to as polycistronic miRNAs, have been described (15). With current miRNA cloning methods, these longer precursor molecules are lost during the multiple size selection steps.

	cDNA clone	MicroRNA-like sequence	Length of homology with entries in Genbank (nt)	Northern blot (nt)	Size of the RT-PCR product minus adaptor (bp)
known	miR-16		22	~22	~20
unknown	4	4	22	~37 ~90 pre*	~35
	7	7	18	~24 ~75 and ~140 pre*	~20
	10	10a	18	-	~20
		10b	28	-	~35
	13	13a	18	-	~20
		13b	29	-	~35
		13c	22	-	~20 pre*?

* pre: precursor?

Table 3. Summary of miRNA-like sequences identified

According to the recommended criteria for miRNA annotation (17), a sequence must fulfill several requirements to be annotated as a miRNA. The first is verification of expression by Northern blots or cDNA cloning of a small RNA ~22 nts. Figure 6 shows results of Northern blot hybridization for clones 4 and 7, where they demonstrate transcripts of distinctly different sizes.

For the five remaining sequences (10a, 10b, 13a, 13b, and 13c) we did not detect any signal on Northern blot which may be due to low abundance level for these molecules in the total RNA

3'-RACE RT-PCR was also performed to identify the presence and size of all seven putative miRNA transcripts. A poly-A tail is added to the 3'-end of the RNA with poly-A polymerase. An oligo-dT containing adaptor is annealed and used for first strand cDNA synthesis. Following reverse transcription, a primer complimentary to the adaptor was used as a reverse RT-PCR primer. The forward primer consisted of the miRNA-like sequence itself (see Figure 7)

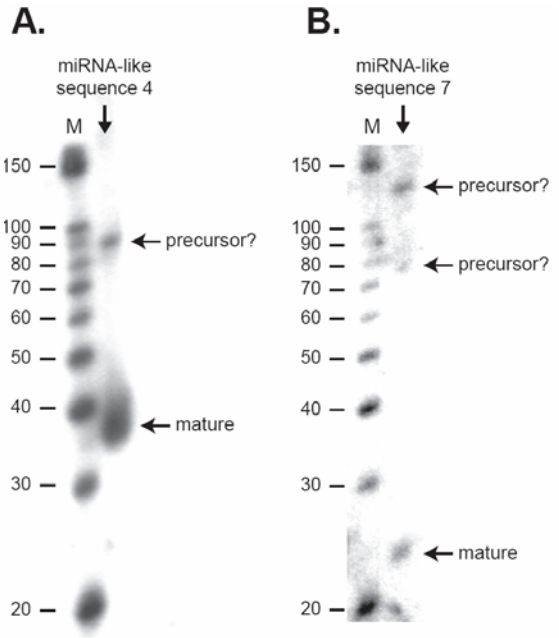

Figure 6. Northern blot analysis of miRNA-like sequences. Synthetic oligonucleotides representing the miRNA-like complimentary sequences in GenBank were used as hybridization probes. 10 nt size markers are indicated. **A**. Results of hybridization of the probe complementary to sequence from clone 4. **B**. Results of hybridization of the probe complementary to sequence from cDNA clone 7.

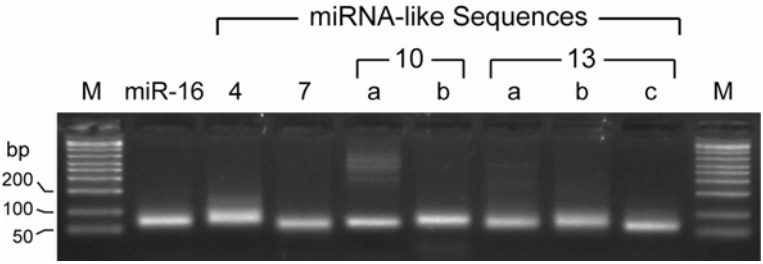

Figure 7. 3' RACE RT-PCR for seven newly identified miRNA-like sequences and miR-16. Numbers on the top of the gel correspond to cDNA clone number. Letters indicate specific sequence found in corresponding clone.

A second criterion for miRNA annotation is evidence of miRNA biogenesis. This typically requires the identification of pre-miRNA stem loop precursors. Stem loops were observed in majority of cDNA clones (data not shown)**.**

For more information and additional data please visit our website at www.systembio.com.

B. Related Products

- **MicroRNA Discovery Kit** (Cat. # RA410A-1)
  Simple and rapid procedure for predominant amplification of miRNA and miRNA-like molecules without size selection ready for cloning without purification. Starting material as little as 0.5 μg.

- **Global MicroRNA Amplification Kit** (Cat. # RA400A-1)
  Simple amplification kit allows cDNA amplification for qRT-PCR and microarray studies from as little as 50 ng of starting total RNA.

- **Full Spectrum™ Complete Transcriptome RNA Amplification Kit** (Cat. # RA101A-1)
  The Full Spectrum RNA Amplification Kit provides an inexpensive method to amplify reverse transcribed RNA in a sequence independent, unbiased, and uniform manner with better representation of 5' end of mRNA sequences. This approach maintains the relative levels of each transcript in the starting mRNA samples—even when using starting amounts of RNA as low as 5ng or when using heavily degraded RNA.

- **Full Spectrum™ MultiStart Primers for T7 IVT** (Cat. # RA300A-2)
  Extract more data from your RNA than currently available primers in nearly all commercially-available T7 IVT kits using Full Spectrum™ technology. Just replace the existing T7 primer with the Full Spectrum™ primers. Compatible with Affymetrix GeneChip® hybridization.

C. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

VI. Licensing and Warranty Statement

Limited Use License

Use of the Pre-made MicroRNA-Enriched cDNAs (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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